A STUDY OF THE EFFECTS OF HYDROSTATIC PRESSURE ON MACROMOLECULAR SYNTHESIS IN *ESCHERICHIA COLI*

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ABSTRACT In cultures of *Escherichia coli* 15 (thymine$^-$, leucine$^-$) which were incubated at high hydrostatic pressures, cell division occurred only at pressures below 430 atm but in a somewhat synchronous fashion at around 250 atm. The rate of leucine-^{14}C incorporation into a macromolecular fraction of the cells diminished to a zero value at about 580 atm and that of uracil-^{14}C incorporation to a zero value at about 770 atm. The rate of thymine-^{14}C incorporation at pressures around 330 atm was that to be expected with a culture in which DNA synthesis is somewhat synchronous. At pressures above 500 atm, thymine-^{14}C was incorporated only over the initial part of the pressure incubation and further incorporation under pressure was not observed no matter how long the duration of the incubation. We present evidence along several lines that the thymine incorporation kinetics reflect an effect of pressure on a locus at the origin (or termination) of a replication of the bacterial chromosome. The recovery of cell division and of the incorporation rates upon release of pressure were found to depend on the magnitude of the pressure and the duration of the pressure incubation.

INTRODUCTION

Living systems encounter high hydrostatic pressures in nature and in the laboratory. In nature organisms have been found living in the depths of the ocean where the hydrostatic pressure is as great as 1,100 atm (1). In the laboratory the incubation of cells and biomolecules at pressures of a few hundred atmospheres results from routine ultracentrifugation (2), and occurs in the process of using a French pressure cell (3). Thus, elucidation of the biological effects of high pressure should be useful in considering life processes in the deep sea as well as in interpreting experiments which unavoidably introduce hydrostatic pressure as a variable.

The effects of hydrostatic pressure on cell division and macromolecular synthesis in cultures of bacteria have been reported from this laboratory (4–6) and others

(7–10). Pressures below 1,000 atm have been found to affect DNA, RNA, and protein synthesis and cell division. We have determined the kinetics of these processes in cultures of *Escherichia coli* 15 T⁻L⁻ (thymine⁻, leucine⁻) at pressures below 1,000 atm during the first few hours of an incubation at high pressure. The results of these experiments and their interpretation are here reported.

MATERIALS AND METHODS

Apparatus

The high pressure system used in these studies is illustrated in Fig. 1 and was constructed by Autoclave Engineers, Erie, Pa. The pressure was generated by an air-driven hydraulic pump (Haskel Engineering and Supply Co., Burbank, Calif.). The air pressure was regulated and the flow of air was controlled by a solenoid valve which in turn was controlled by an electrical contact ring on the face of the pressure gauge. The electrical contact ring could be set to deactivate the solenoid valve when a preset pressure was achieved. The hydraulic fluid was water with an added rust preventative, Immunol (Harry Miller Corporation, Philadelphia, Pa.). An incubation at high pressure was accomplished by putting the culture in a polyethylene bottle and attaching the bottle to an opening on the inside of the cover to the pressure vessel. Then the cover was secured to the pressure vessel and the pressure increased. In order to sample the culture, the pressure had to be released and was reapplied after the sample had been taken. This could be done within five seconds and had no apparent effect on the culture. The cooling experienced by the culture on decompression was compensated by heating on compression. More precisely, a sample was removed by sequentially (*a*) opening the pressure release valve, (*b*) closing the pressure release valve, (*c*) opening the sample valve, (*d*) activating the pump which resulted in the sample being gently forced out of the sample valve, and (*e*) closing the sample valve when a sample size sufficient for analysis was collected. With the sample valve closed, the pump brought the pressure in the system up to a preset value. The temperature was controlled by keeping the pressure vessel immersed in a constant temperature bath.

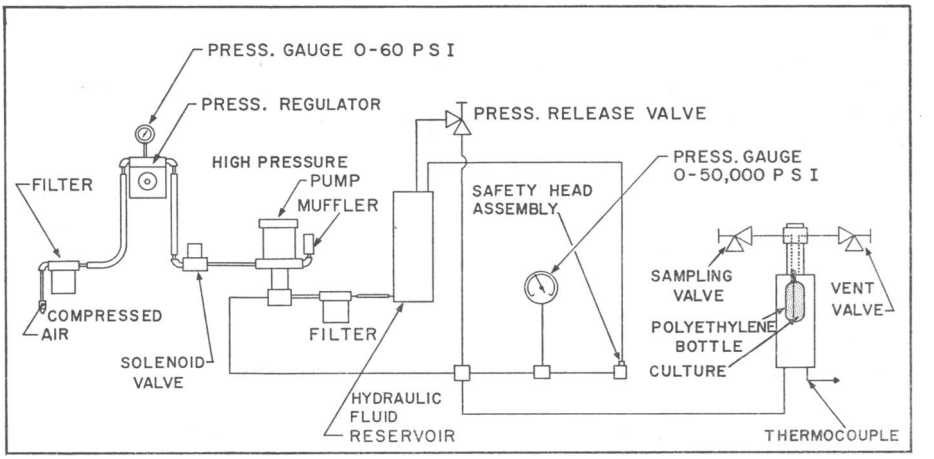

FIGURE 1 Schematic drawing of high pressure apparatus.

Bacterial Strain and Culture Media

A derivative of *Escherichia coli* strain 15 requiring thymine and L-leucine, was used. The minimal medium designated C by Roberts et al. (11) and supplemented with thymine (2 μg/ml), L-leucine (40 μg/ml), and glucose (5 mg/ml) was used. The supplements were autoclaved separately. The cells grew in this medium at 37°C with a doubling time of 46 min, which was determined by using a Coulter counter (Coulter Electronics, Chicago, Ill.) and by plating on agar plates. Cells were grown anaerobically in the supplemented C minimal medium by either adding sodium glutamate (12, 13) and bubbling with nitrogen or bubbling with a N_2—CO_2 (5.2% CO_2) mixture (Matheson Co., Inc., East Rutherford, N. J.). Minimal agar plates were made by incorporating 15 g of agar (Difco Laboratories, Detroit, Mich.) into the formula for supplemented C minimal medium. The agar was autoclaved separately. Nutrient broth agar plates were made from 5 g of NaCl, 8 g of nutrient broth (Difco Laboratories), 5 g of glucose, 15 g of agar, and 1 liter of distilled water. The NaCl and nutrient broth were autoclaved together; the other ingredients were autoclaved individually. Dilutions for plating were made in C minimal medium supplemented with L-leucine and thymine but lacking glucose.

Isotopes

Thymine-2-^{14}C and uracil-^{14}C were purchased from the New England Nuclear Corp., Boston, Mass. D,L-leucine-^{14}C and some thymine-2-^{14}C were purchased from Calbiochem, Los Angeles, Calif. The isotopes were diluted so that labeled cultures had either labeled thymine, labeled L-leucine, or labeled uracil at a concentration of 2 μg/ml, 16 μg/ml, or 40 μg/ml, respectively, and at a specific activity of 10 mc/mmole, 5 μc/mg; or 0.5 μc/mmole, respectively.

Determination of the Amount of DNA, RNA, and Protein Synthesized by Cultures

In a given experiment only one of the above isotopes was introduced into the culture. The amount of incorporation of leucine-^{14}C, uracil-^{14}C, and thymine-^{14}C into a cold 5% trichloroacetic acid (TCA) precipitable fraction of a sample of the culture, was used as a measure (4) of protein, RNA and DNA synthesized, respectively. One ml samples of the culture were taken and added to one ml of 10% TCA at 0°C. The precipitate was collected and washed on 25 mm filters with a pore size of 0.22 μ (Millipore Filter Corp., Bedford, Mass.). The filters were air dried and glued onto planchets. Radioactivity was determined as counts per minute with a Nuclear-Chicago Geiger Counter or a Nuclear-Chicago Gas-flow Counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

RESULTS

The Effects of Pressure on Colony-Forming Ability

The kinetics of the number of colony-forming units in cultures of *E. coli*-15 T^-L^-, which prior to incubation at high pressures had been growing aerobically and logarithmically, were determined at 37°C and at pressures up to 925 atm. Representative data are given in Fig. 2. The ability to divide decreased with increasing pressure of incubation and no cell division occurred at pressures in excess of 500 atm. The kinetics show that the number of colony-forming units in cultures incu-

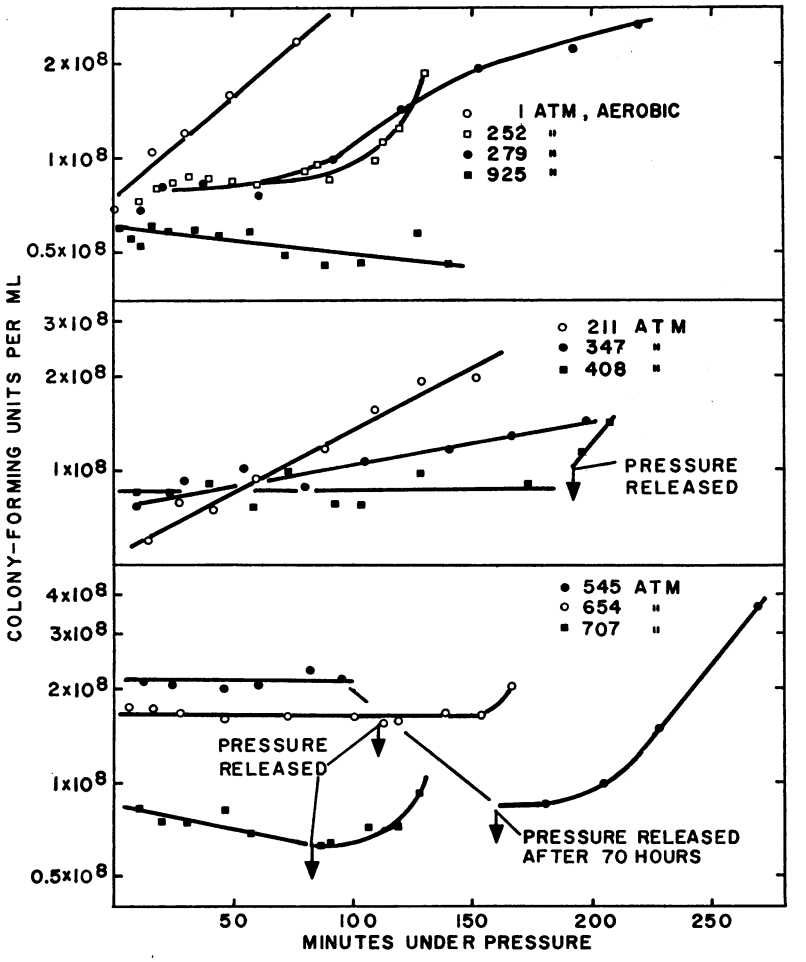

FIGURE 2 The number of colony-forming units in cultures as a function of the duration of the high pressure incubation.

bated around 250 atm increased for at least one division as if the culture were somewhat synchronous. Using a Coulter counter it was determined that the period during which such a culture was not dividing was accompanied by enlargement of the average cell size and that the subsequent period during which the culture was almost synchronously dividing was accompanied by a shift of the average cell size to a smaller value. From the slopes of the straight lines drawn through the points in Fig. 2, exponential growth constants, k (k = ln 2/doubling time), were calculated. At those pressures where the cells were dividing in an apparently synchronous fashion, best straight lines were drawn through the data points and the growth constant calculated is thought of as an average growth constant. These exponential growth constants as a function of pressure are shown in Fig. 13. The line through

these points intersects the pressure axis at about 430 atm and affirms the observation that cells incubated above this pressure could not divide. From the work of ZoBell (1), which was done with *E. coli B* and under experimental conditions somewhat similar to those used in our studies, exponential growth constants were calculated and were found to have a pressure dependence similar to those in our studies.

The data in Fig. 2 show that there was no large lethal effect of pressure for incubations as long as 150 min at pressures up to 925 atm. Longer incubation times at pressures in excess of 500 atm resulted in larger lethal effects. For example, a culture with an initial 2×10^8 colony-forming units per ml, had 8.5×10^7 colony-forming units per ml after a 70-hour incubation at 545 atm, as shown in Fig. 2.

A few observations were made of the number of colony-forming units in cultures on which the pressure had been released and which were being aerated. These recovery phenomena are illustrated in Fig. 2. The number of colony forming units in a culture which had been incubated at 408 atm for about 192 min showed an increase in value immediately following decompression, as seen in Fig. 2. In a culture which had been at 707 atm for about 83 min, a 40 min lag was observed between the time of decompression and that of the resumption in the increase with time of the number of colony-forming units. A similar pattern was observed with a culture which had been incubated at 654 atm for 111 min. Strikingly, the 8.5×10^7 colony-forming units per ml which survived a 70-hour pressure incubation, began increasing with time after a similar 40 min lag.

The Kinetics of Thymine Incorporation

The measure of DNA synthesized in a culture was the amount of incorporation of thymine-2-^{14}C into a cold 5% TCA insoluble fraction of the culture. The isotope was introduced into an aerobic logarithmic culture by collecting the cells on a 25 mm diameter, 0.45 μ pore size Millipore filter, washing them three times with prewarmed medium lacking in thymine, and resuspending the cells in prewarmed medium containing labeled thymine. In some experiments part of the labeled culture was compressed within ten minutes after addition of isotope. In others the pressure incubation was begun after a 50–100 min interval to insure that the filtering procedure preceding the addition of isotope had not introduced artifacts in the subsequent kinetics of thymine incorporation. Figs. 3–5 show the effects of pressure on thymine incorporation at 37, 33, and 25°C, respectively. In all cases the amount of thymine incorporated during an incubation at increased pressure was less than that incorporated by an untreated control. A qualitative summary of the kinetics of thymine incorporation is best discussed by considering the kinetics at 37°C in these pressure intervals. At pressures somewhat below 250 atm, the rate of thymine incorporation increased with time but not on the average as rapidly as in a culture at one atm. At pressures between 250 atm and 450 atm, the kinetics of thymine incorporation by the culture were those of one in which the replication process was somewhat syn-

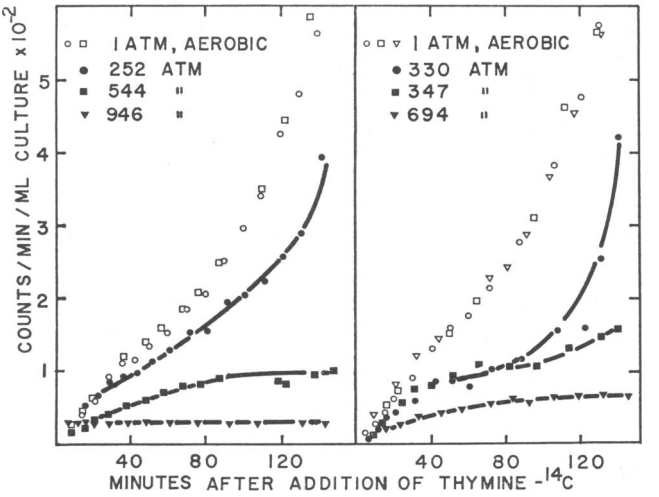

FIGURE 3 Thymine-^{14}C incorporation at 37°C as a function of time after addition of thymine-^{14}C. The incubations at high pressure were begun at the following times: 252 atm, 11 min; 544 atm, 5 min; 946 atm, 4 min; 330 atm, 4 min; 347 atm, 5 min; 694 atm, 5 min.

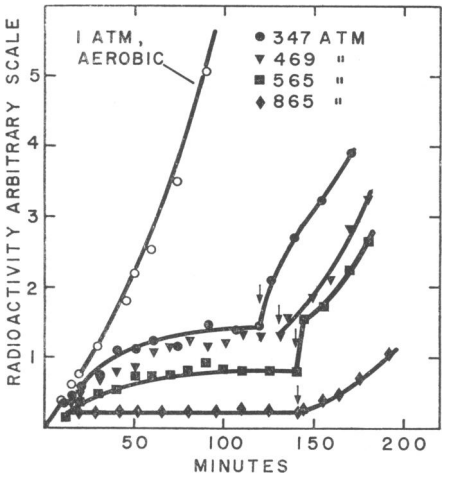

FIGURE 4 Thymine-^{14}C incorporation at 33°C as a function of time after addition of thymine-^{14}C. The arrows indicate the time of pressure release. The incubations at high pressure were begun at the following times: 347 atm, 7 min; 469 atm, 5 min; 565 atm, 7 min; 865 atm, 8 min.

chronous. The synchronous incorporation of thymine was most evident at pressures around 300 atm. In one experiment at 279 atm and 25°C, the synchrony was particularly pronounced (Fig. 5). Upon incubation of cultures at pressures above 500 atm and below 800 atm, the third interval of pressure, an initial period of thymine incorporation occurred after which no further incorporation took place no matter how long the incubation was extended. The amount of thymine incorporated during this initial period diminished with increasing pressure to the extent that incubation of a culture at 946 atm and 37°C, Fig. 3, or at 865 atm. and 33°C, Fig. 4, resulted in

the immediate cessation of thymine incorporation. Thus, a capacity for a limited amount of DNA synthesis remained upon incubation at pressures above 500 atm and somewhat below 900 atm, but DNA synthesis could not be sustained at these pressures. We interpreted this as an effect of pressure on a particular locus of the bacterial chromosome. Some further evidence of this follows.

Aspects of the kinetics of thymine incorporation obtained at high pressure were similar to those following the removal of leucine from a culture of *E. coli* 15 T^-L^-. The effect of removal of a required amino acid on DNA synthesis has been interpreted by Maaløe and Hanawalt (14) and by Hanawalt et al. (15). They demonstrated that the amount of DNA synthesized was consistent with all of the growing points which were present at the time of amino acid removal, completing their synthetic function and with no new growing points being created. The kinetics of thymine incorporation from the incipience of leucine starvation are shown in Fig. 6. The resemblance of the kinetics to, for example, those in the uptake experiment at 544 atm or in the first part of the uptake experiment at 330 atm (Fig. 3), is apparent.

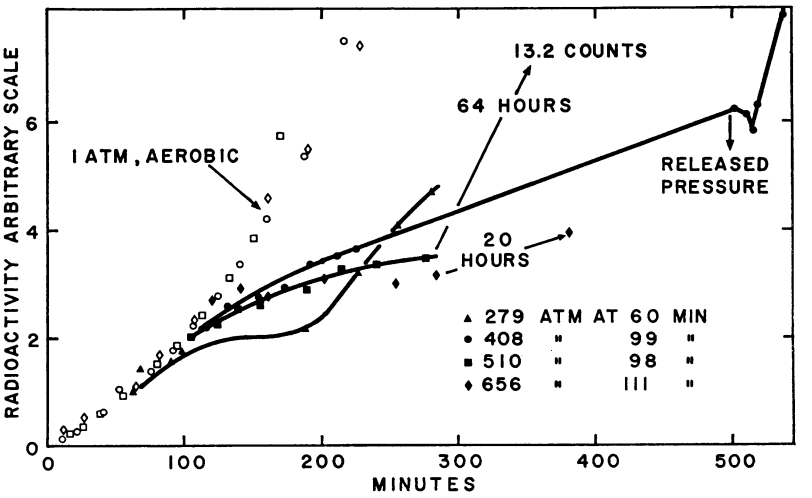

FIGURE 5 Thymine-^{14}C incorporation at 25°C as a function of time after addition of isotope.

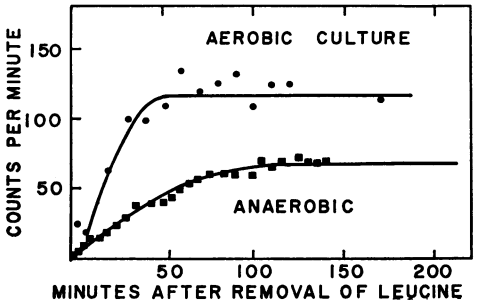

FIGURE 6 The incorporation of thymine-^{14}C at 37°C and at 1 atm following the removal of leucine. The curves drawn through the data have been determined by equation 9.

Another consequence of amino acid starvation is an acquisition of immunity to thymineless death during starvation. If the kinetics of thymine incorporation under pressure reflect processes similar to those occurring subsequent to amino acid starvation, then there should be an acquisition of some immunity to thymineless death in cultures incubated under pressure. The incubation of cells near 358 atm gave incorporation kinetics (see Fig. 3) during the first 80–100 min of incubation, which were similar to those obtained immediately following removal of a required amino acid. A culture incubated at this pressure was sampled at 45, 60, 95, and 120 min after the start of the incubation. Immediately after sampling, each sample was filtered, the cells were washed on the filter with thymineless medium, and then the cells were resuspended in thymineless medium. The resuspensions were sampled at noted times and assayed for colony-forming units on both minimal and nutrient agar plates. As can be seen in Fig. 7, there was an acquisition of some immunity to thymineless death during the incubation at increased pressure. The immunity gained after 60 min of incubation was lost by 120 min of incubation. It should be noted that the cessation of DNA synthesis and acquisition of immunity to thymineless death caused by amino acid deprivation is thought to be the indirect result of the inhibition of protein synthesis; however, protein synthesis is only slightly affected at 358 atm, the pressure at which the acquisition of immunity to thymineless death was observed. This indicates that either a single pressure-sensitive process has been affected at some locus on the chromosome or that the diminished growth rate is cauisng a regulation of DNA synthesis by decreasing the frequency of initiation of replication as proposed by Maaløe and Kjeldgaard (16).

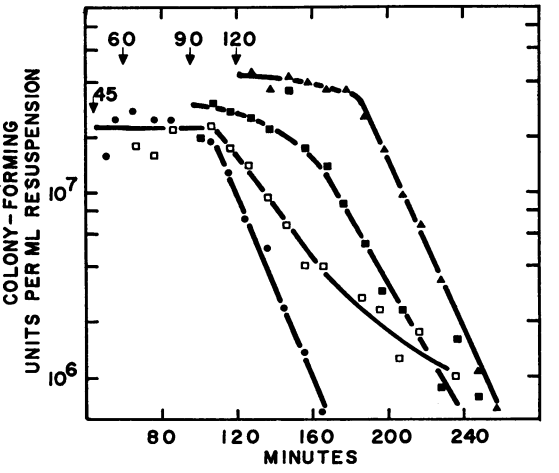

FIGURE 7 At zero time a culture which had been growing aerobically at 37°C and 1 atm, was incubated at 358 atm. At the times indicated by the arrows, the culture was sampled, filtered, and resuspended in thymineless C minimal medium. The resuspension of the 60 min sample (□) shows an acquisition of some immunity to thymineless death.

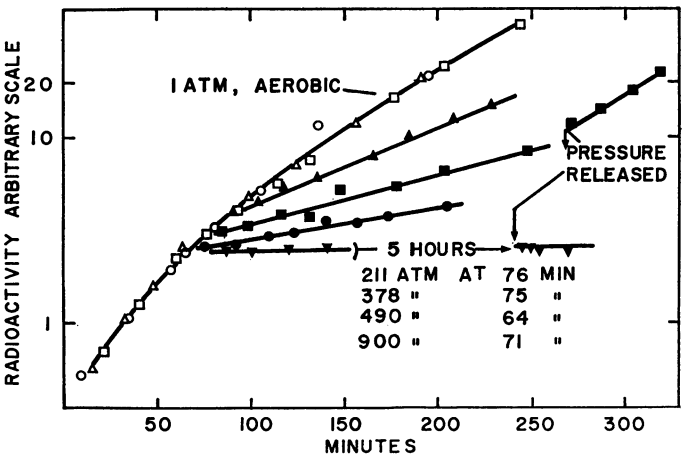

FIGURE 8 The incorporation of leucine-^{14}C as a function of time after addition of isotope at 37°C and at pressures up to 900 atm.

The rate of incorporation of thymine by cultures which had been decompressed and aerated after a 2–3 hr pressure incubation, was comparable to that by untreated cultures shown in Figs. 4 and 5. The fact that recovery occurred immediately could be an indication that one process, highly pressure-sensitive, had been most likely affected. This has been suggested previously (4). Some of the recovery experiments showed a very curious effect. After release of pressure, an immeasurably fast increase in the amount of radioactivity incorporated was observed, as shown by the data in Fig. 4 for the experiment at 565 atm. Possibly, for DNA synthesis to resume so rapidly, everything needed for synthesis must have been exactly where it was to be used. This would reinforce the idea that in preventing DNA synthesis, pressure is affecting one very critical structure or interaction.

The Kinetics of Uracil and Leucine Incorporation

The measure of RNA synthesized in a culture was the amount of incorporation of uracil-^{14}C into a cold 5% TCA insoluble fraction of the culture. The measure of protein synthesized was the amount of incorporation of leucine-^{14}C. The results of experiments at 37°C are shown in Figs. 8 and 9. Plots of the logarithm of the amount of radioactivity incorporated as a function of time gave a linear relationship. The constants obtained from the slopes of such curves are used as a summary of the kinetics at a given pressure. Fig. 13 illustrates the pressure dependence of protein and RNA synthesis as summarized by these constants. The experiments confirm the observation of ZoBell and Cobet (7) and of Pollard and Weller (4) that RNA synthesis continues at pressures where protein synthesis and prolonged DNA synthesis are inhibited.

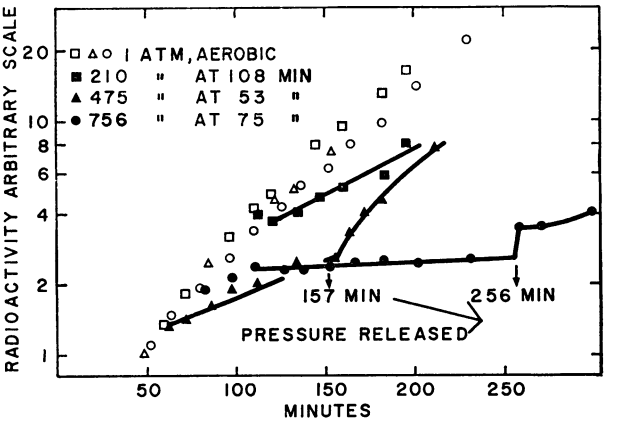

FIGURE 9 The incorporation of uracil-^{14}C as a function of time after addition of isotope at 37°C and at pressures up to 756 atm.

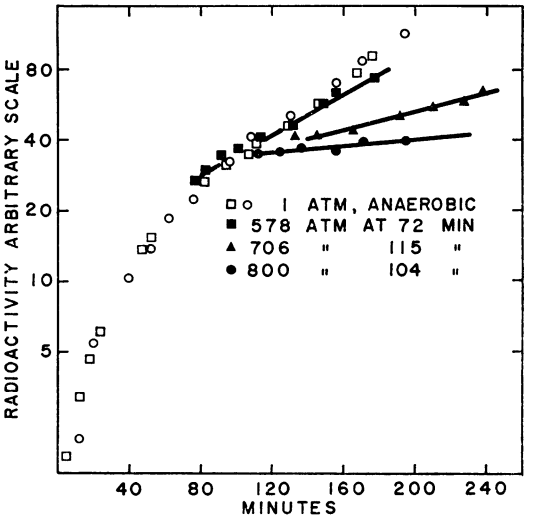

FIGURE 10 The incorporation of uracil-^{14}C as a function of time after addition of isotope at 37°C and at pressures up to 800 atm. Cells were grown anaerobically before being incubated at high pressures.

Anaerobic Experiments

There were two reasons for the anaerobic experiments. (*a*) The procedure of placing an aerobic culture of *E. coli* into a pressure vessel to incubate it at an increased pressure also resulted in the culture being in a potentially anaerobic environment. Although *E. coli* is a facultative aerobe, the quantity of some of its enzymes in the aerobic state is considerably different from that in the anaerobic state (17, 18). Thus the question arose as to whether or not any of the effects of high pressure observed with aerobic cells were due to the cells being in a transition from aerobic to anaerobic growth. (*b*) The aerobic cultures contained dissolved oxygen which at high pressure has been shown to adversely affect some biochemical and cellular processes

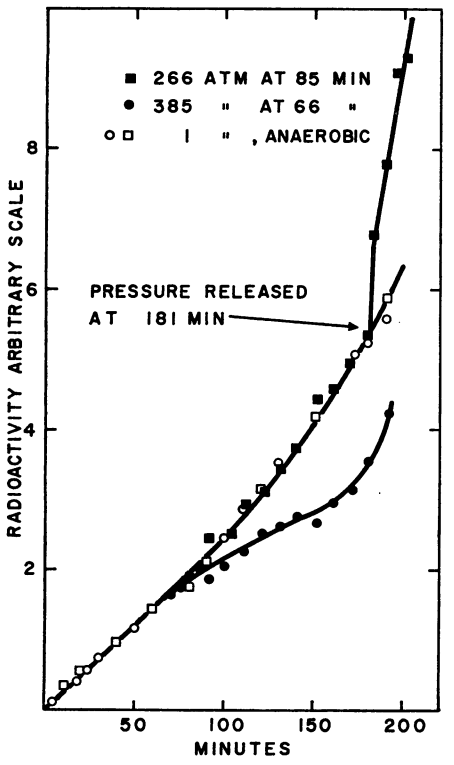

FIGURE 11 The incorporation of thymine-^{14}C as a function of time after addition of isotope at 37°C and at 266 and 385 atm. Cells were grown anaerobically before being incubated at high pressures.

(19–20). This posed the question of whether dissolved oxygen had any role in the effects observed with aerobic cells.

To gain an idea of the magnitude of any possible effects due to these two reasons, we repeated the studies on thymine and uracil incorporation using anaerobic cells. Some of the results are shown in Figs. 10 and 11 in which it is evident that anaerobic cells were not as sensitive as aerobic cells to high pressures, in that effects comparable to those obtained with aerobic cells occurred at higher pressures with anaerobic cells. However, the interesting features of the kinetics obtained with aerobic cultures under pressure were also obtained with anaerobic cultures. Thus, the apparently synchronous incorporation of thymine and the incorporation of uracil at the higher pressures were observed with anaerobic cells as well as with aerobic cells.

DISCUSSION

These experiments show what happens to certain processes in cultures of *E. coli* during the first few hours of incubation at high pressures. The processes observed reflect cell division and the synthesis of DNA, RNA, and protein. At pressures up to 430 atm, cell division occurred but at a frequency which diminished with increasing pressure. Above 430 atm, cells in the culture did not divide. The pattern of divi-

sion in cultures which were incubated around 250 atm, was one of a somewhat synchronous culture. The interesting feature of this behavior was that the thymine incorporation kinetics, reflecting DNA synthesis, exhibited a similar synchronous behavior at pressures also around 250 atm. Indeed, the periods of cell division were accompanied by thymine incorporation and the periods of no cell division were attended with no incorporation. A plausible hypothesis is that a process related to both cell division and DNA synthesis had been affected by the pressure. A rate-limiting role for such a process could explain the synchronous behavior at pressures around 250 atm and its inhibition at higher pressures could explain the failure of cells to divide when incubated above 430 atm. The pressure-sensitive process related to both division and DNA synthesis does not appear to involve energy metabolism since protein and RNA synthesis are only slightly affected whereas cell division is blocked by pressures above 430 atm. An effect on the chemistry, in a quantitative sense, of cell wall formation does not appear to be likely since filaments can form above pressures of 430 atm (7). Since DNA synthesis, albeit for a limited duration, can occur under incubation at pressures somewhat above 700 atm, effects of lesser pressures cannot be thought of as being primarily on the polymerase activity of a growing point. The part of the bacterial chromosome involved with the origin (and, hence, termination) of a replication is the locus for a number of processes whose pressure sensitivity could be giving the above results. At this locus, for example, the free ends of the newly synthesized strands of the daughter chromosomes must be joined, the newly formed chromosomes separated, new replications started, and, perhaps, interactions between the growing point and the membranes established (16). Four consequences of such a pressure-sensitive process at this locus were examined.

First, if such a pressure-sensitive process were located near this locus, then the kinetics of thymine incorporation by cultures which are under pressure should be similar to those by cultures from which leucine has been removed. The rationale for this is that removal of a required amino acid from a culture results in the inhibition of DNA synthesis because of the failure to reinitiate DNA synthesis at the origin of replication (15). Aspects of the kinetics of incorporation during incubation under high pressure were indeed similar to those following removal of leucine, as can be seen from a comparison of the data in Fig. 6 with that in Figs. 3, 4, or 5. Since protein synthesis is occurring in the cultures at these pressures, the failure to reinitiate replication can be thought of as a direct effect of the pressure.

Second, inhibition of DNA synthesis by a block at the origin of a replication has also been shown to be accompanied with an acquisition by the culture of immunity to thymineless death (15). Accordingly, if the growing points in a culture under pressure are accumulating at their origin of replication because a process at its locus has become rate limiting, then an immunity to thymineless death should be observed during this accumulation. During an incubation of a culture at 358 atm, an acquisition followed by a loss of some immunity to thymineless death was observed (Fig. 7).

Acquisition of immunity to thymineless death by all of the cells in a culture during a pressure incubation would be difficult to demonstrate since DNA synthesis promptly resumes, and hence, immunity is rapidly lost on decompression.

A third consequence of a pressure-sensitive process at the origin of a replication is that the amount of radioactivity incorporated by cultures between the start of an incubation at high pressure and the point at which the rate of thymine incorporation reaches a zero value, should correspond to an amount of DNA that would be synthesized by an initially exponential population of cells if the formation of all new growing points were stopped. This amount of DNA can be calculated from the probability density function, $f(x)$, for the location of growing points along the chromosomes of cells in an exponential population of cells.

$$f(x) = (\ln 2)\, 2^{1-x}, \tag{1}$$

where x is the location of the growing point with the origin at $x = 0$ and the terminus at $x = 1$. This function is taken from Sueoka and Yoshikawa (22). It follows that the average position of a growing point will be at 0.44 units from the origin. Hence, if no new growing points are created and all existing growing points complete their polymerase function, then there will be a 39% increase in the amount of DNA in a culture. We made calculations from the kinetics of thymine incorporation to see if the increment observed between the amount of radioactivity incorporated at the time of application of pressure and that when a zero rate of thymine incorporation was achieved, corresponded to a 39% increase in the amount of DNA in a culture. To make this calculation, we began with the fact that in a population of cells whose number is increasing exponentially, the amount of DNA at some time, t, $[\mathrm{DNA}]_t$, is increasing according to

$$[\mathrm{DNA}]_t = [\mathrm{DNA}]_0 \exp(kt), \tag{2}$$

where $[\mathrm{DNA}]_0$ is the amount of DNA at time zero and k is the growth constant of the cells, $k = (\ln 2)/(\text{doubling time})$. If at time zero a compound which is strictly a precursor of the DNA is added, then the amount of radioactivity in the DNA after a time, t, will be proportional to the amount of DNA synthesized in that time. Thus,

$$[\mathrm{cpm}]_t = K'\{[\mathrm{DNA}]_0 \exp(kt) - [\mathrm{DNA}]_0\} \tag{3}$$

or

$$[\mathrm{cpm}]_t = K[\exp(kt) - 1], \tag{4}$$

where K' is a proportionality constant and K is the product of K' and $[\mathrm{DNA}]_0$. This equation applies if the population of cells was growing exponentially both at and subsequent to the time at which radioactivity was added and if the effects of

pools can be ignored. The procedure used to introduce labeled thymine into the culture resulted in approximately a five min thymineless incubation of the culture during the filtering which was done to remove unlabeled thymine. Consequently, some disruption of the exponential nature of the cultures was probably encountered and thus equation 4 is not strictly valid, particularly for the early part of the kinetics. From equation 2 it can be shown that the interval of time, Δt, which must elapse for a 39 % increase in the amount of DNA in a culture, is:

$$\Delta t = (\ln 1.39)/k. \tag{5}$$

The increment of radioactivity incorporated between any time t and Δt (equation 5) later is:

$$\Delta[\text{cpm}] = (0.39)K \exp(kt). \tag{6}$$

At the start of a high pressure incubation, the untreated control and the culture under pressure have the same amount of DNA and radioactivity. After a time, Δt, the untreated control will have 39 % more DNA. The determination of the change in the radioactivity corresponding to this 39 % increase can be made, by using Δt, either graphically from the data or with equation 6. The results of a graphical determination of the amount of radioactivity corresponding to a 39 % increase in the amount of DNA in the control cultures are shown in Table I. Also shown, in each case, is the increment in radioactivity between that at the start of a high pressure incubation and that when a zero rate of incorporation occurred. The calculations indicate that at pressures between 330 and 550 atm, the calculation is consistent with the idea of a pressure-sensitive site localized at the origin (or terminus) of a replication. During incubation at pressures above 550 atm, the calculations are not consistent with this idea of a pressure-sensitive locus probably because pressure is also affecting the polymerase activity of a growing point in addition to affecting termination (or initia-

TABLE I

P	T	$k \times 10^2$*	Δt‡	Δcpm§ (calculated)	Δcpm (observed)
atm	°C	min^{-1}	*min*		
330	37	1.04	32	92	90
544	37	1.35	24	60	55
544	37	1.08	30	100	90
690	37	1.45	23	45	30
456	33	0.98	34	130	140
578	33	0.87	38	140	70
690	25	0.75	44	425	395

* Determined by plating on agar plates.
‡ Equation 5.
§ Calculated graphically from the untreated control thymine uptake data and Δt.

tion) of replication. The effect on the polymerase activity is most obvious in the experiment at 946 atm in which the thymine incorporation ceases immediately with the start of the pressure incubation.

We can use the probability density function of Sueoka and Yoshikawa (22), equation 1, to test in a fourth way the idea that no new replications are initiated upon incubation of a culture at some of the high pressures. We can use this function to predict the kinetics of thymine-^{14}C incorporation by an exponentially growing culture in which initiation of new replications is suddenly prevented. Let ρ represent the rate of movement of one growing point along a chromosome and assume ρ is identical for all growing points. Assume that all of a sudden new growing points are prevented from being formed. The number of growing points at this time is N_0 and these are distributed between the origin ($x = 0$) and the terminus ($x = 1$) of the chromosome according to equation 1. Let t represent the time following the cessation of growing point formation. In a time, t, those growing points between $x = 1$ and $x = 1 - \rho t$ will have completed their DNA synthesis. Thus, the number of growing points in a culture is diminishing according to the following relationship:

$$N_t = N_0 - N_0 \int_{1-\rho t}^{1} (\ln 2) 2^{1-x} dx. \tag{7}$$

We can assert that the rate of thymine-^{14}C incorporation, $d[\text{cpm}]_t/dt$, is proportional to the product of the rate of movement of a growing point and the number of growing points; that is,

$$d[\text{cmp}]_t/dt = C\rho \left[N_0 - N_0 \int_{1-\rho t}^{1} (\ln 2) 2^{1-x} dx \right], \tag{8}$$

where C is a proportionality constant. We note that $d[\text{cpm}]_t/dt \to 0$ as $\rho t \to 1$ and that $d[\text{cpm}]_t/dt \to CN_0\rho$ as $t \to 0$.
Integrating equation 8,

$$[\text{cpm}]_t = 2CN_0\rho t - (CN_0/\ln 2)(2^{\rho t} - 1) \tag{9}$$

and we note that when $t = 1/\rho$, $[\text{cpm}]_t = [\text{cpm}]_{\max}$ which is,

$$[\text{cpm}]_{\max} = CN_0[2 - (1/\ln 2)]. \tag{10}$$

Equation 10 and $d[\text{cpm}]_t/dt$ at $t = 0$ can be used to calculate values of CN_0 and ρ from data. These values and equation 9 determine the kinetics of thymine incorporation if the model applies. The kinetics of thymine incorporation subsequent to the removal of leucine from cultures are shown by the points in Fig. 6. The curves drawn through these points were calculated from values of CN_0, ρ, and equation 9. Thus, the equation is adequate in describing the kinetics of thymine incorporation by leucine-requiring cells subsequent to leucine starvation. Maaløe and Hanawalt

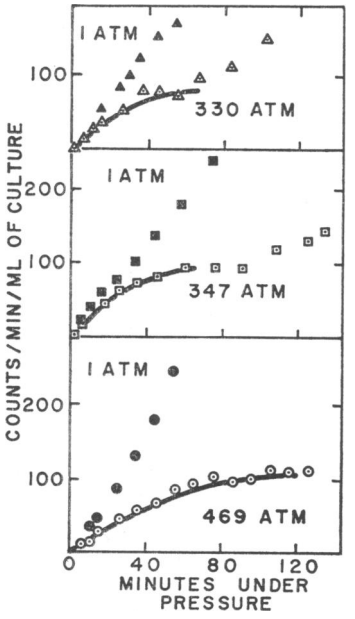

FIGURE 12 The amount of thymine-^{14}C observed to have been incorporated is shown by the points. The solid curves were calculated as described in the text using equation 9. The 330 and the 347 atm experiments were at 37°C and the 469 atm experiment was at 33°C.

TABLE II

P	T	ρ
atm	°C	(*Chromosome lengths/min*)
330	37	1.4×10^{-2}
347	37	1.22×10^{-2}
347	37	1.5×10^{-2}
442	37	1.12×10^{-2}
442	37	1.27×10^{-2}
347	33	1.05×10^{-2}
415	33	0.97×10^{-2}
544	33	0.41×10^{-2}
680	33	0.24×10^{-2}

(14) have shown that removal of a required amino acid can be thought of as causing the cessation of creation of new growing points, yet permitting the completion of already initiated rounds of replication. The kinetics of thymine incorporation by cultures incubated at high pressures were similar in many cases to those obtained subsequent to leucine starvation. We were able to fit data (which was obtained with cultures incubated between 330 and 680 atm) with calculated values of ρ, CN_0, and equation 9.

Some representative fits are shown in Fig. 12. With data from pressures less than 500 atm, equation 9 is applicable from the start of the pressure incubation to the time the rate of incorporation of thymine reaches a zero value. This is probably because, at pressures below 500 atm, initiation of new rounds of replication has been delayed

but not completely inhibited. Hence, the delay in the pressure interval of 330–500 atm is long enough to permit completion of rounds of replication which were initiated prior to the application of pressure and long enough for this completion to occur in the absence of the formation of new rounds of replication. Incubations above 500 atm at 37°C result in the complete inhibition of initiation of new growing points. But the incorporation kinetics at pressures somewhat above 600 atm most likely also reflect, and increasingly so with increasing pressure, an effect of pressure on the polymerase activity of the growing point as well as effects on protein synthesis, RNA synthesis, and perhaps other cellular activities.

It is also apparent that equation 10, together with the condition that $(d[\text{cpm}]/dt) \rightarrow CN_0\rho$ as $t \rightarrow 0$, can be used to estimate the rate of growing point movement. Values of ρ calculated in this way are shown in Table II.

The relationship between the inhibition of DNA synthesis and the induction of filament formation by incubation at high pressures was found by ZoBell and

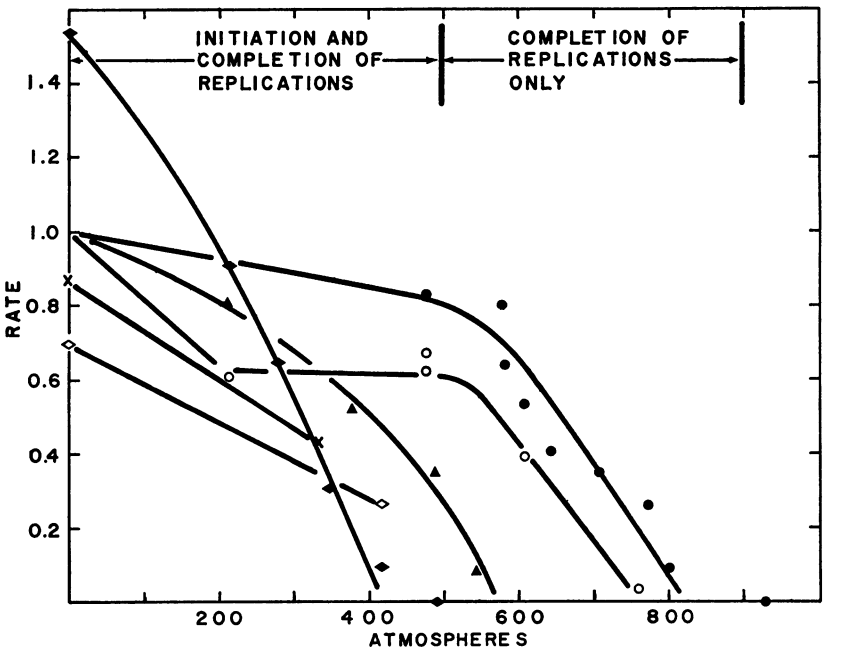

FIGURE 13. A summary of the effects of pressure on the rates of some of the processes observed: ●, the rate of uracil-^{14}C incorporation at high pressure relative to that at 1 atm, 37°C and anaerobic conditions; ○, the relative rate of uracil-^{14}C incorporation, 37°C and aerobic conditions; ▲, the relative rate of leucine-^{14}C incorporation, 37°C and aerobic conditions; the exponential growth constant, k, for cells at 37°C (◆), 33°C, (×) and 25°C (◇), all at aerobic conditions. The pressure interval of 0–500 atm is indicated as being compatible with growing point initiation and DNA synthesis. Above 500 atm, initiation of new replications is prevented and DNA synthesis by growing points which existed prior to the pressure incubation is inhibited with increasing pressure.

Cobet (7). They pointed out that inhibition of DNA synthesis was probably the cause of filament formation. Our evidence expands on this conclusion and makes it plausible that the effect on replication is at a locus near the end (or, hence, the start) of a replication.

The question arises whether the process being affected by pressure is inactivated in some cells or inhibited in all of the cells in the culture. The fact that the rate of thymine incorporation recovered instantly to the control rate after incubations of up to three hours duration at pressures as high as 946 atm, tends to rule out a mechanism involving inactivation in some cells as opposed to inhibition in all of the cells.

ZoBell and Cobet (7) described the content of DNA, RNA, and protein in cells that had been incubated at pressures up to 500 atm. Besides revealing the above mentioned inhibition of DNA synthesis at high incubation pressures, their results showed a disproportionate increase in the amount of RNA per cell compared to that of DNA and protein, as a function of increasing pressure. Pollard and Weller also found that the rate of incorporation of uracil was almost unaffected compared to that in an untreated control at pressures where protein precursors were not incorporated at all. The results reported here on protein and RNA synthesis are in qualitative agreement with the above conclusions.

Since the amount of radioactivity, incorporated either as uracil-^{14}C or leucine-^{14}C, increased exponentially with time, plots of the logarithm of the radioactivity incorporated versus the time gave a linear relationship. From such plots constants were calculated. Fig. 13 displays these constants as a function of pressure. It can be seen that at a pressure of 600 atm RNA synthesis is occurring in the absence of protein synthesis. The similarity of this effect of pressure to that observed with relaxed control mutatants (23), has been pointed out by ZoBell and Cobet (7).

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